Spring 2015

The Characterization of Monoclonal Antibodies Specific to HPV31 L1 Protein

Colin Coleman
Colin.Coleman@Colorado.EDU

The Characterization of Monoclonal Antibodies Specific to HPV31 L1 Protein

Colin R. Coleman

Department of Molecular, Cellular, and Developmental Biology

University of Colorado at Boulder

Defended March 18, 2015

Thesis Advisor

Dr. Robert Garcea, Department of Molecular, Cellular, and Developmental Biology

Committee Members

Dr. Jennifer M. Martin, Department of Molecular, Cellular, and Developmental Biology

Dr. J. Harrison Carpenter, Department of Ecology and Evolutionary Biology

Abstract:

The research project described in this thesis details the characterization of monoclonal antibodies designed to react with the L1 protein of Human Papillomavirus type 31, as measured by specific laboratory assays. These antibodies were tested for reactivity against HPV31 L1 proteins in Western blots, enzyme-linked immunosorbant assays (ELISAs), and pseudovirus neutralization assays. The characterization of these antibodies is an important step in the production of second-generation HPV vaccines, where the antibodies would be used during the design and manufacturing process.

The results of this research present characterization data for each of 48 different monoclonal antibodies produced as to their reactivity in Western blots, ELISAs, and pseudovirus neutralization assays. A wide range of reactivity across all antibodies was observed for each assay. After reviewing the completed data, reactive candidate monoclonal antibodies for each assay were determined and will be immediately utilized in various quality control steps in second-generation vaccine development in the Garcea Lab, expediting the production of more stable and cost-effective multivalent vaccines.

1. Introduction

Over the past few decades, cervical cancer has been recognized as one of the leading causes of cancer deaths in women. On a worldwide scale, this cancer is the fourth most common and contributes to the fourth most cancer deaths in women (Stewart and Wild, 2014; Chaturvedi, 2010). Cervical cancer is also the eighth most common cancer in women in the United States, and recent medical statistics list five-year survival rates at 68% (Howlander et al., 2014). In developing nations, where routine screenings and vaccinations are less predominant to nonexistent, these survival statistics are even lower and the cancer even more prevalent (Brewer and Fazekas, 2007; Clifford, 2003; Smith et al., 2007).

Human Papillomavirus (HPV) has been shown to be a necessary factor in cervical cancer development and progression (Wallboomers et al., 1999). This dsDNA virus has icosahedral structure composed of a protein outer shell and an inner genome that codes for viral replication and structural proteins. The shell is made up of 72 pentameric subunits, with each subunit comprising of five interconnected proteins known as L1 (Schwarz et al., 1985). These pentamers contribute greatly to infection, as they interact with the human cell surfaces to cause endocytosis and consequently cell infection and viral replication (Schiffman et al., 2007).

Cervarix® and Gardasil®, the two HPV vaccines that are currently on the market, are designed to prevent initial infection by the virus, and subsequent development of cervical cancer. Both work extremely well in preventing infection, but unfortunately are currently only applicable in developed countries (Hillman et al., 2012; Giuliano et al., 2012; Harper et al., 2004). This scope is limited because the method of production used with these vaccines makes them both expensive to produce and unstable to transport, two critical factors preventing their use in the developing countries where they are needed most (Roden and Wu, 2006; Kane et al., 2006). In

addition to the suboptimal production method, Gardasil is only designed to protect against the four most common types of HPV, specifically types 6, 11, 16, and 18, and Cervarix only against 16 and 18. While these HPV types account for the majority of cervical cancer cases, the fact remains that several other types, especially types 31 and 45, are equally as carcinogenic despite being not as common as the others ("FDA", 2010; Bosch et al., 2007). With these considerations in mind, work has already begun on second-generation HPV vaccine development, intended to address these concerns.

In order for future HPV vaccines to be utilized in developing nations, they need to be designed in a way that allows them to remain stable at high temperatures, due to the lack of reliable cold chain transit in such countries. Currently, HPV vaccines are composed of virus-like particles (VLPs), in addition to adjuvants ("FDA", 2010; Inglis, Shaw, and Koenig, 2006). VLPs are hollow, reconstructed capsids of viruses, in this case HPV (Chen et al., 2000; Kimbauer, 1992). Since VLPs have no genetic material, they do not pose a threat to humans upon injection yet still allow for the immune system to generate antibodies against the antigens they present and therefore are the most accurate simulation of an infection for the body's immune system to react against. Unfortunately, VLPs in vaccine solutions are structurally unstable and require near-constant refrigeration in order to remain viable.

Recent research has shown that a comparable immune response can be initiated with L1 protein pentamers in contrast to the complete viral shell, sparking the potential for a more stable and cost-effective vaccine option (Yuan et al., 2001; Schellenbacher et al., 2009). L1 pentamers maintain their antigenicity with more stability than the complex VLP structure, and therefore could avoid refrigeration altogether. In addition, L1 proteins are much easier to produce in a laboratory setting than VLPs. Currently, the self-assembling capsid pentamers for VLPs are

produced in insect cell lines ("FDA", 2010). L1 pentamers are easily produced and purified using *E. coli* bacteria, potentially reducing the cost of production as well as increasing the chance of producing usable proteins. To increase the protective range of these second-generation vaccines, L1 proteins of other high-risk HPV types will need to be added to the vaccine solution as well. One such type, HPV31, was the focus of this project due to its high carcinogenicity and absence in current vaccines.

Vaccine development requires constant quality control, so it is important to develop reactive antibodies to HPV31 L1 in order to maintain consistency in the development and production process, prompting the initiation of this research project. Protein-specific antibodies are important elements for protein characterization, therefore having antibodies known to bind HPV31 L1 protein in different assay-specific formats is vital to proper testing (Patel et al., 1989; Smith et al., 1983). To create such an antibody set, antibody-producing cells were isolated, immortalized, and subcloned from immunized mice challenged with HPV31 L1 antigen at NeoClone (Madison, WI, USA), creating a wide range of monoclonal antibodies, named such due to their shared lineage from an original mouse cell (Roseto et al., 1984; Waldmann, 1991). These antibodies were tested in Western blots, enzyme-linked immunosorbant assays (ELISAs), and pseudovirus neutralization assays to determine if they were reactive to the HPV31 L1 protein in those formats. Antibodies found to most successfully react to the protein in these characterization assays were then approved to be used to test for the presence of HPV31 L1 protein in future vaccine development protocols and experiments.

2. Materials and Methods

Aside from section 2.2, all research was conducted in the Garcea Lab on the University of Colorado Boulder campus. Section 2.2, pertaining to monoclonal antibody development and production, was completed by NeoClone (Madison, WI, USA) upon sending them purified HPV31 L1 protein.

2.1 HPV31 L1 Production

HMS174 *E. coli* bacterial cells were heat transformed with a plasmid containing a kanamycin resistance gene and an HPV31 L1 encoding gene coupled to a *lac* operator. Transformed colonies were selected for using Luria broth (LB) media plates with kanamycin and allowed to incubate overnight at 37°C. Single colonies were inoculated into 3 ml of Terrific broth (TB) containing 50 ug/ml kanamycin diluted 1:1000 and grown for 6 hours at 30°C with shaking. Two hundred microliters of this culture was then inoculated into 50 ml of TB containing 50 ug/ml kanamycin and grown overnight at 30°C with shaking. The following day, 2 ml of the 50 ml culture was inoculated into each of 6 baffel flasks containing 500 ml TB with 50 ug/ml kanamycin for 3 liters total culture. Flasks were incubated at 37°C with shaking until the optical density at 600 nm reached 4.0, at which point the flasks were chilled to 25°C in a cold water bath. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Research Products International, Mount Prospect, IL, USA) was added to the culture to a final concentration of 0.2 mM to initiate HPV31 L1 expression. The induced culture was incubated at 25°C with shaking until the optical density at 600 nm reached 8.0. The bacterial cells were pelleted by centrifugation at 5,800 x g for 15 minutes at 4°C and stored at -20°C overnight.

For purification, the frozen bacteria pellets were resuspended in a total of 300 ml homogenizing buffer (200 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM PMSF, 10% glycerol, 5 mM DTT + 2 protease inhibitor tablets [Roche]) and run twice through a Panda homogenizer (GEA Niro Soavi, Parma, Italy) at ~800-1000 barr. The resulting lysate was centrifuged at 22,000 x g for 30 minutes at 4°C, pelleting out insoluble bacterial proteins and cell debris and recovering the desired L1 proteins in the soluble fraction (S1). Next, the S1 was chromatographed using a 300 ml Q Sepharose Fast Flow (QFF) (GE Healthcare, Piscataway, NJ, USA) column equilibrated with Running buffer (50 mM Tris, 200 mM NaCl, 10% glycerol, 5 mM DTT, pH to 8.1) to purify the L1 protein from other undesired bacterial proteins. The L1 protein present in the QFF flow-through (FT) were precipitated out of solution by slowly adding solid ammonium sulfate to reach 30% saturation. The ammonium sulfate solution was incubated for 2 hours at 4°C with stirring in order to precipitate the L1 protein. The solution was centrifuged at 13,000 x g for 30 minutes and the resulting pellet (AS-P) containing the precipitated L1 proteins was stored at 4°C overnight.

The L1 pellet was resuspended in Running Buffer (25 mM NaCl, 50 mM Tris pH 8.5, 5 mM DTT, 10% glycerol, pH 8.5) and passed through the Panda homogenizer at ~500 barr to ensure complete resuspension of the protein in solution. After homogenization, the solution was centrifuged at 13,000 x g for 20 minutes and the soluble fraction containing the L1 protein was saved. The conductivity of the soluble fraction was adjusted to match that of the Running buffer using no-salt buffer (50 mM Tris pH 8.5, 5 mM DTT, 10% glycerol, pH 8.5) and pH was adjusted to 8.5. The fraction was loaded onto a 75 ml Q Sepharose High Performance (QHP) (GE Healthcare, Piscataway, NJ, USA) column equilibrated with Running Buffer. The L1 protein was eluted from the column over an increasing concentration gradient from 25 mM to 1

M NaCl Running Buffer. The eluted fractions, as well as samples from the steps in the purification, were analyzed on 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to determine the presence of the L1 protein based on its specific size (52 kD). Fractions containing L1 protein according to both the chromatograph and the SDS-PAGE results were then pooled, quantified, flash frozen in liquid nitrogen and stored at -80°C.

2.2 Monoclonal Antibody Production

Purified HPV31 L1 protein was sent to NeoClone (Madison, WI, USA) for monoclonal antibody production. Three total fusions were conducted, with quality control ELISAs being run first by NeoClone. Fusions were then sent to Boulder for confirmation of positive clones in our ELISAs. A total of 48 positive antibody-producing hybridoma cell lines were selected. Antibodies from these hybridomas were purified using protein affinity chromatography, labeled as to their cell line, and aliquots were shipped back to Boulder for analysis.

2.3 Western Blots

The HPV31 L1 monoclonal antibodies were tested for reactivity in Western blot assays. Effectiveness was judged as the ability to accurately signal the presence of HPV31 L1 protein in a sample while minimizing cross-reactive signaling of similar, yet distinct, antigens. A sample size of 0.1625 ug of purified HPV31 L1 protein with added 3x Sample Buffer + β-mercaptoethanol (BME; Sigma-Aldrich, St. Louis, MO, USA) was separated by 10% SDS-PAGE (150 V, 500 mA, 75 minutes), along with similar amounts HPV16 L1 protein and mouse polyomavirus (MPyV) VP1 protein (viral shell protein) in separate lanes as cross-reactivity indicators. The gel was washed three times for 10 minutes in Transfer Buffer (250 mM Tris, 2

M glycine). The size-separated proteins in the gel were transferred onto a nitrocellulose membrane using a Hoefer IE70 Semi-dry Transfer Unit (Amersham Biosciences, Madison, WI, USA) at 15 V and 500 mA for 45 minutes. Once the viral proteins were transferred to the membrane, the membrane was blocked overnight with a 5% milk in Tris-buffered Saline Tween20 (TBST) solution.

The following day, the block buffer was removed and the membrane washed three times for 5 minutes each with TBST. A primary antibody solution of purified HPV31 L1 monoclonal antibody diluted 1:1000 in TBST was then added to the membrane and incubated at room temperature, rocking for one hour. After the hour, the primary antibody solution was removed, the membrane washed three times for 5 minutes each with TBST, and then a secondary antibody solution consisting of goat-αMouse-alkaline phosphatase antibody (Promega, Madison, WI, USA) diluted 1:5000 in TBST. After an hour, the secondary antibody solution was removed and the membrane was washed three times for 5 minutes each with TBST. A developer solution was made, consisting of BCIP (5-bromo-4-chloro-3'-inolyphosphate-p-toluidine; Fisher Scientific, Pittsburgh, PA, USA) and NBT (nitro-blue tetrazolium chloride) (Fisher Scientific, Pittsburgh, PA, USA) as the major developing reagents in a 1X dilution of alkaline phosphatase developing solution (0.4 M Tris, 1 M NaCl, 0.2 M $MgCl_2$). This solution was applied to the membrane and allowed to react until visible banding appeared, at which point the developer was removed, the membrane rinsed with water to stop the reaction, and the membrane allowed to air dry. If after 10 minutes no banding was detected, the membrane was rinsed with water and allowed to air dry.

2.4 Enzyme-Linked ImmunoSorbent Assays

The HPV31 monoclonal antibodies were tested for reactivity in ELISAs. Three serial dilutions of HPV31 L1 protein in 1x Phosphate-buffered Saline (PBS) were added to a Polysorp 96 well plate (Nunc, Naperville, IL, USA), so that each of the three experimental rows had 0.25 ug, 0.13 ug, and 0.06 ug of protein in their wells, respectively, with a final volume of 50 ul per well. A fourth row containing 50 ul PBS only was also implemented as a negative assay control. The protein was allowed to bind the plate overnight at 4°C.

The next day, the plate was washed three times using 50 ul Wash Buffer (PBS + 0.05% Tween20) in order to remove any non-bound L1 protein. One hundred microliters per well of Block Buffer (5% non-fat dry milk, 0.05% Tween20 in PBS, pH 7.4) was added in order to block non-specific binding to the plate. The plate was incubated with Block Buffer for one hour at 37°C.

Block Buffer was removed and 50 ul/well of a primary antibody solution consisting of purified HPV31 monoclonal antibody diluted 1:1000 in Block Buffer was added. A set of wells using primary αL1 antibodies and a set of wells with primary αV5 tag monoclonal antibodies against plated tagged HPV16 L1 protein were used as positive controls. A set of wells using primary αV5 tag antibodies against plated HPV31 L1 protein and a set of wells using primary αV5 tag antibodies against plated MPyV VP1 protein were used as negative controls. The plate was incubated for one hour at 37°C, and the plate was again washed three times for 5 minutes each with 50 ul Wash Buffer. A 50 ul/well secondary antibody solution consisting of anti-mouse HRP (horseradish peroxidase) conjugated IgG secondary antibodies (Promega, Madison, WI, USA) diluted 1:5000 in Wash Buffer was added to the plate and incubated at 37°C for one hour.

After allowing the secondary antibodies to bind the monoclonal antibodies, the plate was washed three times with 50 ul Wash Buffer. Room temperature TURBO 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Thermo Scientific, Waltham, MA, USA) was added 50 ul/well to the plate. After allowing ten minutes for color to develop, 50 ul/well 1 M sulfuric acid was added to stop the reaction. Absorbance values were determined in an ELx808 absorbance reader (BioTek, Winooski, VT, USA) at a wavelength of 450 nm.

2.5 Pseudovirus Neutralization Assays

The HPV31 L1 monoclonal antibodies were tested for reactivity in pseudovirus neutralization assays. The antibodies were judged based on their ability to neutralize pseudoviruses, reconstructed HPV31 viruses containing a reporter plasmid as their genetic material, and prevent them from infecting other cells. 293TT simian kidney cells were cultured in supplemented Dulbecco's Modified Eagle's Medium (DMEM; supplemented with 1% PBS, 1% Antimicrobial-Antimycotic [Sigma-Aldrich, St. Louis, MO, USA], 1% MEM [Life Technologies, Grand Island, NY, USA], 1% Glutamax [Life Technologies, Grand Island, NY, USA]), with routine passaging at 90% confluency. When at approximately 80% confluency, the cells were washed with PBS, trypsinized and counted. Cells were centrifuged at 2000 x g for 5 minutes and then resuspended in supplemented DMEM minus phenol red (DMEM-PR) to a concentration of $3x10^5$ cells/ml. One hundred microliters per well of this resuspension was added to the internal wells of a Greiner CellStar 96-well cell culture plate (Sigma-Aldrich, St. Louis, MO, USA). One hundred and fifty microliters per well of DMEM was added to the external wells of the plate in order to prevent evaporation from the internal wells. The plate was covered and incubated for 2-5 hours at 37°C.

Six serial dilutions (1:80, 1:320, 1:1280, 1:5120, 1:20480, 1:81920) of the HPV31 L1 monoclonal antibodies in supplemented DMEM-PR were created in a round-bottom 96 well plate (Fisher Scientific, Pittsburgh, PA, USA), with a final volume of 50 ul/well for the lowest dilution and 60 ul/well for all other dilutions.

One hundred and twenty microliters per well of HPV31 pseudoviruses containing Secreted Embryonic Alkaline Phosphatase (SEAP) reporter plasmids (Inviragen, Ft. Collins, CO, USA) diluted 1:250 in DMEM-PR were added to the wells a new round-bottom 96 well-plate using siliconized pipet tips. Thirty microliters per well from the antibody dilution plate was added to the wells of the pseudovirus plate using siliconized tips. The negative neutralization control was α-Bovine Papillomavirus (BPV) antibody diluted 1:50 in DMEM-PR. The positive neutralization control was 1 mg/ml heparin. Wells containing only 293TT cells in DMEM-PR and wells containing 293TT cells in DMEM-PR with a 1:250 dilution of pseudovirus were used as background controls. Neutralization was allowed to proceed for one hour at 4°C, after which 100 ul/well of the pseudovirus/antibody mix was gently added to the plated 293TT cells and the plate was incubated at 37°C for 72 hours.

Following the 72 hour incubation, 50 ul of the cell media from each of wells was aspirated and placed in a new round-bottom 96 well plate (Fisher Scientific, Pittsburgh, PA, USA), which was centrifuged for 5 minutes at 1000 x g for clarification. Forty-five microliters per well of 1x Dilution Buffer from a Great Escape SEAP Chemiluminescence Detection Kit 2.0 (Clontech, Mountain View, CA, USA) was added to the wells of a Costar white 96-well optiplate (Fisher Scientific, Pittsburgh, PA, USA), to which 15 ul/well of the clarified supernatant was then added. The optiplate was wrapped in coverfoil and incubated at 65° for 30 minutes. After incubation, the optiplate was chilled for 3 minutes on ice, then brought to room temperature

before 60 ul/well of room-temperature chemiluminescence substrate 4-methylumbelliferyl phosphate (MUP) from the Detection Kit was added. The optiplate was left to incubate in darkness at room temperature for one hour. After incubation, the optiplate was read for chemiluminescence values in a Synergy 2 Multi-mode plate reader (BioTek, Winooski, VT, USA).

3. Results

Results reported below reflect characterization data generated at the Garcea Lab at University of Colorado, Boulder campus. These experiments were run from January 2014 through February 2015.

3.1 Western Blots

All 48 HPV31 L1 monoclonal antibodies were tested for reactivity in Western blotting (Fig. 1). Western blot data was qualitative and was based upon the amount of banding that was developed on the blot. The monoclonals were organized on an arbitrary scale of 0-4 based on their Western blot reactivity, with 0 meaning no banding was detected and 4 meaning that excessive banding was detected, including in the cross-reactivity lanes (Fig. 2).

Of the 48 monoclonal antibodies tested, 30 showed at least some banding indicative of HPV31 L1 recognition. The remaining 18 antibodies, designated Tier 0 (T0) produced no visible banding, indicating that the monoclonals were unable to recognize protein in the Western blot. The 11 antibodies designated Tier 1 (T1) had very faint banding in the HPV31 L1 lane and no visible banding in the cross-reactivity lanes. Tier 2 (T2) consisted of four antibodies that exhibited stronger banding that T1 antibodies, but less cross-reactivity than the next tier. The 11

antibodies in Tier 3 (T3) showed strong, visible HPV31 L1 banding, but also exhibited slight cross-reactivity with HPV16 L1 protein. Four antibodies were designated Tier 4 (T4), due to their over-reactive nature in Western blotting. These T4 antibodies not only bound to all three structural proteins, but they also bound to extraneous degradation proteins that remained in the HPV31 L1 sample.

3.2 ELISAs

All 48 monoclonal antibodies were tested for reactivity in ELISAs (Fig. 3). Using two 96-well plates per assay, 20 monoclonal antibodies were tested at a time. Reactivity was determined by absorbance values of the monoclonal antibody wells, with higher absorbance values reflecting recognition of the HPV31 L1 protein. A set of wells using primary αL1 antibodies and a set of wells with HPV16 L1 protein bound to the plate and primary αV5 tag monoclonal antibodies were used as positive controls. The αL1 antibodies recognized HPV31 L1 on the plate and the αV5 tag antibodies recognized the V5 tag attached to the HPV16 L1 protein. A set of wells using primary αV5 tag monoclonal antibodies against plated HPV31 L1 protein and a set of wells using primary αV5 tag monoclonal antibodies against plated MPyV VP1 protein were used as negative controls. The αV5 tag antibodies were unable to recognize both the tagless HPV31 L1 protein and the polyomavirus VP1 protein. Absorbance values ranged from 0 RAU (Relative Absorbance Units) (4D11H7, 0.06 ug/well L1) to 0.396 RAU (4D3C6, 0.25 ug/well L1).

3.3 Pseudovirus Neutralization Assays

Each of the 48 monoclonal antibodies was tested for reactivity in pseudovirus neutralization assays (Fig. 4). Using two 96-well plates per assay, all data was completed in three successful assays. Reactivity was determined by luminescence values, with lower values reflecting higher neutralization. Fractional neutralization values were defined as the difference between the αBPV value and the HPV31 L1 monoclonal antibody values, divided by the difference between the αBPV value and the heparin value. Percent neutralization was defined as 100 x the fractional neutralization value. This data was plotted over the course of the six serial dilutions of antibodies.

4. Discussion

This data has determined several strong candidate HPV31 L1 monoclonal antibodies for use in Western blots, ELISAs, and pseudovirus assays (Table 1). Subtle changes in antibody paratopes led to varying degrees of HPV31 L1 reactivty in each of the different experimental formats. Characterizing these antibodies has illustrated specific antibodies that effectively recognize HPV31 L1 protein in each of the three assays tested.

Western blotting determined which antibodies were reactive to denatured HPV31 L1 protein. Visible banding on the blots occurred due to the reaction of the developer to the alkaline phosphatase enzyme attached to the secondary antibody: the enzyme hydrolyzes the BCIP in the developer into a form that NBT is able to oxidize, which creates a dark blue diformazan precipitate that is visible on the membrane as banding. If the monoclonal antibody being tested was reactive to the viral proteins on the membrane (HPV31 L1, HPV16 L1, or the VP1), then the secondary αMouse-AP antibodies would be able to react with the bound monoclonal antibody.

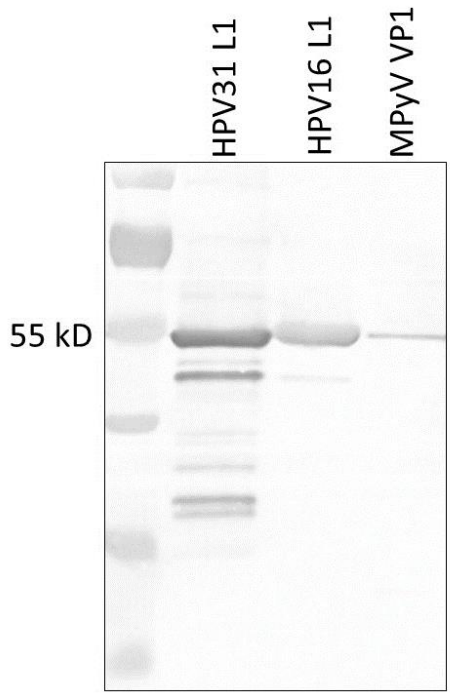

Fig. 1. Layout of HPV31 monoclonal antibody Western blot. Monoclonal antibodies were tested as to their effectiveness in binding HPV31 L1 proteins in Western blotting. HPV16 L1 and mouse polyomavirus (MPyV) shell protein VP1 were included to monitor antibody cross-reactivity. An individual Western blot assay was run for each of the 48 monoclonal antibodies (Antibody 3F5F6H8 shown).

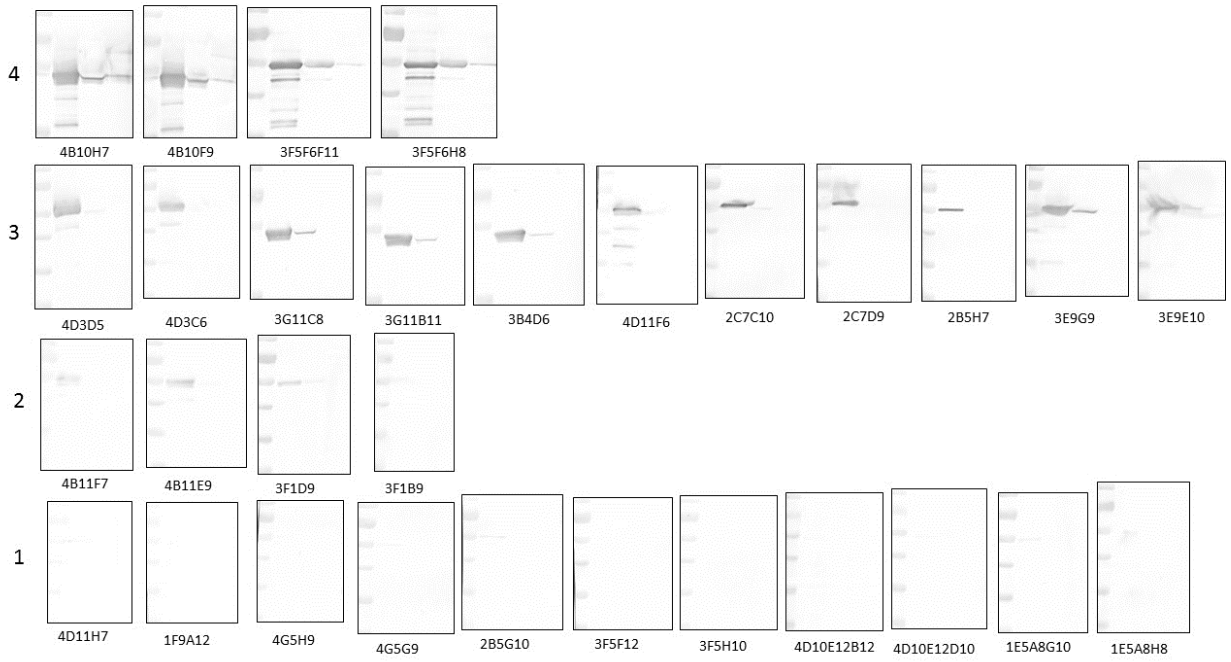

Fig. 2. Compilation of Western blots for HPV31 monoclonal antibodies. See Fig. 1 for assay layout. Blots were organized by an arbitrary scale of 0-4 based on the amount of banding observed after developing. Not shown are blots at tier 0 which showed no discernable banding whatsoever.

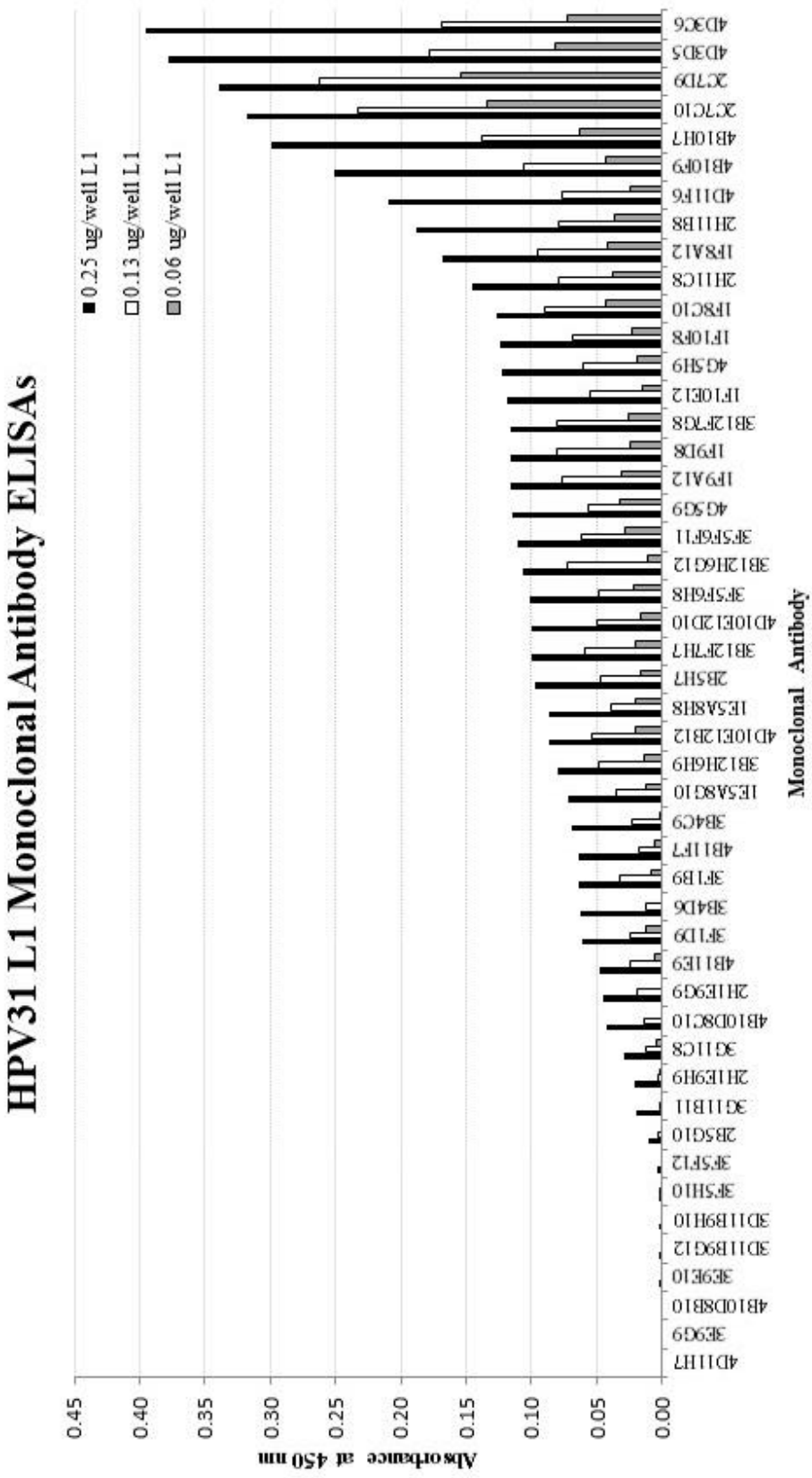

Fig. 3. Compiled HPV31 L1 monoclonal antibody ELISA absorbance data. Each monoclonal antibody was run in duplicate over three dilutions of plated HPV31 L1 protein: 0.25 ug/well, 0.13 ug/well, and 0.06 ug/well. Absorbance RLU values were generated by the plate reader set for 450 nm wavelength end point absorbance. Antibody data was organized by increasing RLU values at 0.25 ug/well plated HPV31 L1 protein.

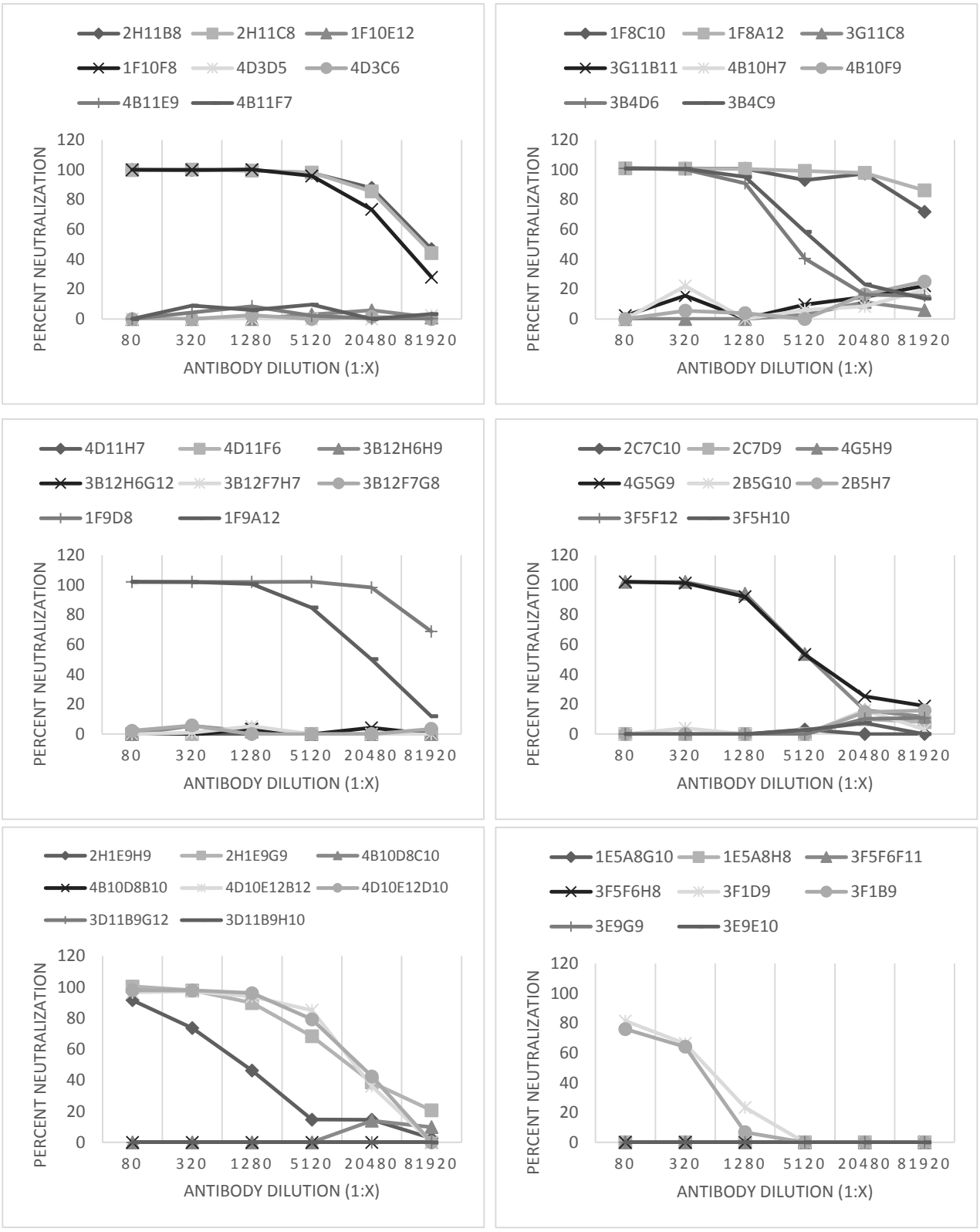

Fig. 4. Compiled HPV31 L1 monoclonal antibody pseudovirus neutralization data. Each antibody was run over six serial dilutions with a constant 1:250 dilution of pseudovirus stock. Percent neutralization values were generated by comparing luminescence values of the antibody wells to a positively neutralizing heparin control.

Monoclonal Antibody	Western Blots	ELISAs (absorbance)			Pseudovirus Neutralization Assays (%Neutralization)					
		HPV31 L1 plated (ug/well)			Monoclonal Antibody Dilution					
Subtype:	Tier #	0.25	0.13	0.06	1:80	1:320	1:1280	1:5120	1:20480	1:81920
1E5A8G10	1	0.073	0.035	0.012	0.00	0.00	0.00	0.00	0.00	0.00
1E5A8H8	1	0.087	0.040	0.021	0.00	0.00	0.00	0.00	0.00	0.00
1F10E12	0	0.118	0.056	0.015	0.00	0.00	0.00	2.93	5.81	0.96
1F10F8	0	0.124	0.068	0.024	100.00	99.96	100.19	95.88	73.41	27.94
1F8A12	0	0.168	0.0955	0.042	100.98	100.72	100.62	99.26	97.96	86.21
1F8C10	0	0.126	0.0895	0.0425	100.98	100.91	100.75	93.14	97.42	71.87
1F9A12	1	0.116	0.077	0.031	102.10	101.94	100.68	84.83	50.22	11.96
1F9D8	0	0.116	0.081	0.024	102.09	102.09	102.09	102.10	98.31	68.86
2B5G10	1	0.011	0.003	0	0.00	4.03	0.00	0.00	16.04	3.07
2B5H7	3	0.097	0.0465	0.017	0.00	0.00	0.00	0.00	14.77	15.88
2C7C10	3	0.319	0.234	0.134	0.00	-0.29	0.00	3.06	0.00	0.00
2C7D9	3	0.340	0.263	0.155	0.00	0.00	0.00	0.00	10.03	8.09
2H11B8	0	0.188	0.079	0.037	99.93	99.83	99.60	97.89	87.85	46.75
2H11C8	0	0.145	0.079	0.038	99.74	100.12	99.41	98.22	85.53	44.17
2H1E9G9	0	0.045	0.0185	0	100.37	97.77	89.69	68.28	38.60	20.63
2H1E9H9	0	0.0205	0.0025	0.001	91.51	73.52	46.25	14.70	14.61	2.55
3B12F7G8	0	0.117	0.080	0.026	2.24	5.85	0.00	0.00	0.00	3.53
3B12F7H7	0	0.100	0.059	0.021	0.00	1.05	5.25	0.00	0.00	0.00
3B12H6G12	0	0.1065	0.073	0.011	0.00	0	3.42	0.00	4.25	0.00
3B12H6H9	0	0.0805	0.048	0.014	0.00	5.68	1.71	0.00	0.00	0.00
3B4C9	0	0.069	0.023	0.0005	100.82	100.64	95.18	58.46	23.17	13.63
3B4D6	3	0.062	0.013	0	100.98	100.23	90.99	40.47	16.19	15.52
3D11B9G12	0	0.0025	0	0	0.00	0.00	0.00	0.00	0.00	0.00
3D11B9H10	0	0.003	0	0	0.00	0.00	0.00	0.00	0.00	0.00
3E9E10	3	0.002	0.000	0.000	0.00	0.00	0.00	0.00	0.00	0.00
3E9G9	3	0.000	0.000	0.000	0.00	0.00	0.00	0.00	0.00	0.00
3F1B9	2	0.064	0.032	0.009	75.97	64.14	6.79	0.00	0.00	0.00
3F1D9	2	0.061	0.025	0.013	81.41	66.30	23.40	0.00	0.00	0.00
3F5F12	1	0.004	0	0	0.00	0.00	0.00	0.00	10.34	10.87
3F5F6F11	4	0.110	0.062	0.029	0.00	0.00	0.00	0.00	0.00	0.00
3F5F6H8	4	0.101	0.049	0.022	0.00	0.00	0.00	0.00	0.00	0.00
3F5H10	1	0.003	0.0015	0	0.00	0.00	0.00	2.76	7.49	0.00
3G11B11	3	0.0195	0.002	0	2.22	15.63	0.10	9.55	14.75	22.21
3G11C8	3	0.0295	0.012	0.004	0.00	0.00	0.00	2.83	11.36	5.86
4B10D8B10	0	0	0	0	0.00	0.00	0.00	0.00	0.00	0.00
4B10D8C10	0	0.043	0.014	0	0.00	0.00	0.00	0.00	13.91	9.84
4B10F9	4	0.2505	0.106	0.043	0.00	5.55	3.87	0.00	16.46	25.13
4B10H7	4	0.299	0.138	0.063	0.00	22.12	0.00	6.00	8.40	19.54
4B11E9	2	0.048	0.025	0.006	0.00	4.34	8.50	2.16	0.32	0.24
4B11F7	2	0.064	0.018	0.006	0.00	8.94	5.98	9.58	0.00	3.32
4D10E12B12	1	0.0865	0.054	0.0205	96.51	97.17	93.83	85.04	36.21	0.00
4D10E12D10	1	0.1	0.05	0.0165	97.88	97.84	96.05	79.21	42.47	0.00
4D11F6	3	0.21	0.076	0.024	0.00	0.00	3.12	0.00	0.00	0.00
4D11H7	1	0	0	0	0.00	0.00	0.00	0.00	0.00	0.00
4D3C6	3	0.396	0.169	0.072	0.00	0.00	2.45	0.00	1.50	0.00
4D3D5	3	0.378	0.178	0.082	0.00	0.00	2.43	2.69	6.25	6.08
4G5G9	1	0.114	0.057	0.033	102.13	101.34	92.14	53.35	25.41	18.85
4G5H9	1	0.123	0.060	0.019	102.03	102.07	94.13	53.77	15.99	11.52

Table 1. Compiled data for all 48 monoclonal antibodies. Data from Western blotting, ELISAs, and Pseudovirus neutralization assays for each of the antibodies is shown.

This binding would then provide the alkaline phosphatase for the developer reagents to react with, creating the visible banding. If the monoclonal antibody was unreactive to the viral proteins, then the secondary antibodies would have nothing to bind to, causing alkaline phosphatase to be absent upon developing and resulting in a lack of banding.

In the resultant Western blots, there was a gradient over which the monoclonal antibodies were reactive to the L1 protein on the nitrocellulose membrane, leading to the subjective T0-T4 scaling system devised to organize the data. T0 antibodies were not reactive to the protein, and were quickly disregarded for future Western blotting. T1 antibodies loosely, albeit specifically, recognized the HPV31 L1 protein in the assay, but not strongly enough to be considered useful for future Western blot experiments.

Antibodies designated T2 showed HPV31 L1 recognition stronger than was seen in T1 antibodies, but these antibodies were not as reactive as T3 antibodies and were disregarded for future Western blots.

T3 antibodies were the first to show significant recognition of HPV31 L1, but there was also some reactivity to the HPV16 L1 and MPyV VP1 cross-reactivity controls. The genetic similarity between the two types of HPV L1 protein explains this cross-reactivity, and the fact that the HPV31 reactivity was stronger than the HPV16 reactivity maintained the usefulness of these antibodies for future experimental purposes.

T4 antibodies reacted to much more than the HPV31 L1 protein, including both cross-reactivity lanes as well as degradation products found in the HPV31 sample. This nonspecific binding made most T4 antibodies not fit for future experimentation with Western blots. Once all Western blot data had been collected, the 3G11C8 (T3) and 3F5F6H8 (T4) antibody subtypes were chosen to be kept maintained for future Western blotting. 3F5F6H8, a T4 antibody, was

chosen despite its cross-reactivity because of the distinct banding for each of the lanes. While 3G11C8 will be used more for quality control, having a known cross-reactive antibody in 3F5F6H8 was thought to have some potential use.

ELISAs determined which monoclonal antibodies were reactive to conformationally accurate HPV31 L1 bound to a plate. Higher absorbance values were indicative of successful monoclonal antibody reactivity to HPV31 L1 protein. This is because such reactivity was necessary for subsequent secondary antibody binding, which provides the HRP enzyme for TMB oxidation and consequently causes the color change in the solution. Monoclonal antibodies unreactive to the HPV31 L1 created wells where secondary antibodies were unable to bind and unable to initiate color change upon TMB addition.

The ELISA data showed an interesting trend of reactivity, that when organized by increasing absorbance the antibodies exhibited a gradual increase in reactivity until the last ten antibodies, where the numbers increased dramatically. This trend is another illustration of how the subtle changes in antibody paratopes can lead to great differences in HPV31 L1 reactivity. Antibody subtypes 2C7D9 and 4D3C6 were picked for future use in vaccine developmental ELISAs due to their high levels of absorbance reflecting strong reactivity to plate-bound HPV31 L1 protein.

An observation worth noting is that antibodies with strong affinity in the Western blots were not necessarily as successful in ELISAs. While several subtypes proved to be efficient in both experiments (4B10F9, 4B10H7, 2C7C10, 2C7D9, 4D3D5, 4D3C6), many T3 and T4 Western blot antibodies had very low absorbance values in ELISAs, indicative of poor HPV31 L1 recognition. The reason for this lies in the structural integrity of the L1 proteins in the assays. In Western blotting, the proteins are denatured by SDS in order to pass through the acrylamide

gel effectively. In ELISAs, the L1 proteins remain in their natural conformation bound to the plate. These differences create two unique HPV31 L1 epitopes to recognize, resulting in some antibodies being reactive to the denatured version in Westerns and other reactive to the stable version in ELISAs. Such variations further highlight the significance of this characterization project.

The pseudovirus neutralization assays were fundamentally different than the Western blots and ELISAs; where the latter experiments tested how well HPV31 monoclonal antibodies recognized HPV31 L1 alone, the pseudovirus assays tested how well the monoclonals reacted to a conformationally accurate HPV31 viral capsid in solution. During the 72 incubation period, HPV31 pseudoviruses that were not neutralized by HPV31 monoclonal antibodies were able to infect the 293TT cells with their SEAP plasmids. The abundance of SV40 Large T Antigen in the 293TT cells initiated the expression of SEAP due to its link to an SV40 origin sequence; this enzyme was then secreted by the cell into the supernatant. Pseudoviruses combined with positively neutralizing monoclonal antibodies were unable to infect the 293TT cells, and therefore no SEAP was exported to the supernatant of those wells. If the reactivity was strong, then the antibodies prevented infection of the 293TT cells by the pseudovirus, a scenario similar to how human antibodies react to a real viral infection.

By running the assay with serial dilutions of antibodies, not only were we able to see if the monoclonals were able to recognize and neutralize the pseudovirus, but we also observed how quickly neutralization values dropped off as the concentration of antibodies decreased. These dilutions were important because some monoclonal antibodies were able to maintain high neutralization even at low concentrations, proving to be more effective at recognizing fully formed HPV31 capsids. We chose to maintain the 1F9D8 and 1F8A12 antibody subtypes for

future HPV31 pseudovirus assays due to their consistently high neutralization of pseudovirus across all antibody dilutions.

5. Conclusion

Current vaccines to prevent HPV infection, while effective, are expensive to manufacture and difficult to stably transport, limiting their availability in developing nations where they are needed most. By using HPV L1 proteins as the primary antigen in second-generation vaccines, production costs and transportation issues have the potential to be lowered to a point where the vaccines could be effectively distributed in these disadvantaged countries. In order to incorporate these HPV L1 proteins into new vaccines, reactive antibodies to the L1 protein must be developed for quality control to be maintained. HPV31, a carcinogenic HPV type not protected against in current vaccines, was chosen as the focus of this thesis project. HPV31 L1 proteins were purified from bacterial cells, monoclonal antibodies were produced against the antigenic protein, and those antibodies were characterized for reactivity in Western blots, ELISAs, and pseudovirus neutralization assays, three assays important in the vaccine development process.

The results of this project are straightforward, yet instrumental for second generation HPV vaccine research. While HPV types 16 and 18 are still the most prevalent in cervical cancer cases worldwide, types such as HPV31 are still impactful in the cancer statistics and worth studying from a public health standpoint (Muñoz et al., 2003; Tota et al., 2011). Second-generation multivalent HPV vaccines being designed in the Garcea lab will be produced to protect against HPV31, and therefore having effective in-house antibodies that recognize HPV31 L1 is an important preparatory step in the developmental process.

The medical success of first generation HPV vaccines relieves some efficacy concerns of future development, allowing for more innovation and fine-tuning in second generation vaccine production. For example, the Randolph lab at the University of Colorado is currently working on a process based on previous research that will safely lyophilize, or freeze dry, HPV vaccine solutions so that they can be transported with more stability and at a lower shipping cost to developing countries (Carpenter et al., 1997; Hassett et al., in submission). When combined with the use of L1 proteins over VLPs as the vaccine antigen, second generation vaccines have the potential to be extremely cost-effective and travel-efficient. The L1 monoclonal antibody characterization scheme used here for HPV31 can easily be reproduced for other high-risk carcinogenic HPV types as well, facilitating their inclusion in future vaccines. With all of these vaccine development elements in place, cervical cancer rates due to HPV infection stand to drop dramatically not only in developed nations but developing ones as well, and the research reported herein will contribute to the foundation of such an endeavor.

Works Cited

Bosch, F X et al. “The Causal Relation between Human Papillomavirus and Cervical Cancer.” *Journal of Clinical Pathology* 55.4 (2002): 244–265. Print.

Brewer, N.T.Fazekas, K.I. “Predictors of HPV Vaccine Acceptability: a Theory-Informed, Systematic Review.” *Prev Med*. 2007; 45:107–114.

Carpenter, John F., et al. "Rational Design of Stable Lyophilized Protein Formulations: Some Practical Advice." *Pharmaceutical research* 14.8 (1997): 969-975.

Chaturvedi, Anil K. "Beyond Cervical Cancer: Burden of Other HPV-Related Cancers Among Men and Women." *Journal of Adolescent Health* 46.4 (2010): S20-26.

Chen, Xiaojiang S., et al. "Structure of Small Virus-like Particles Assembled from the L1 Protein of Human Papillomavirus 16." *Molecular Cell* 5.3 (2000): 557-567.

Clifford, G M et al. “Human Papillomavirus Types in Invasive Cervical Cancer Worldwide: A Meta-Analysis.” *British Journal of Cancer* 88.1 (2003): 63–73. PMC.

"FDA Licensure of Bivalent Human Papillomavirus Vaccine (HPV2, Cervarix) for Use in Females and Updated HPV Vaccination Recommendations from the Advisory Committee on Immunization Practices (ACIP)." *Morbidity and Mortality Weekly Report* 59.20 (2010): 626-29. JSTOR.

Giuliano, Anna R. et al. “Efficacy of Quadrivalent HPV Vaccine against HPV Infection and Disease in Males.” *The New England Journal of Medicine* 364.5 (2011): 401–411. PMC. Web. 26 Feb. 2015.

Hassett, Kim J. et al. “Eliminating the Cold Chain: A Highly-Thermostable, Adjuvanted HPV Vaccine” *European Journal of Pharmaceutics and Biopharmaceutics* (in submission).

Hillman, Richard J. et al. “Immunogenicity of the Quadrivalent Human Papillomavirus (Type 6/11/16/18) Vaccine in Males 16 to 26 Years Old.” *Clinical and Vaccine Immunology* : CVI 19.2 (2012): 261–267. PMC. Web. 26 Feb. 2015.

Howlader, N et al. “SEER Cancer Statistics Review, 1975-2011.” *Bethesda, MD: National Cancer Institute* (2014): 1975-2011.

Inglis, Stephen, Alan Shaw, and Scott Koenig. "HPV Vaccines: Commercial Research & Development." *Vaccine* 24 (2006): S99-S105.

Kirnbauer, R., et al. "Papillomavirus L1 Major Capsid Protein Self-assembles into Virus-Like Particles that are Highly Immunogenic." *Proceedings of the National Academy of Sciences* 89.24 (1992): 12180-12184.

Kane, Mark A., et al. "HPV Vaccine Use in the Developing World." *Vaccine* 24 (2006): S132-S139.

Munoz, Nubia, et al. "Epidemiologic Classification of Human Papillomavirus Types Associated with Cervical Cancer." *New England Journal of Medicine* 348.6 (2003): 518-527.

Patel, D., P. S. Shepherd, J. A. Naylor, and D. J. Mccance. "Reactivities of Polyclonal and Monoclonal Antibodies Raised to the Major Capsid Protein of Human Papillomavirus Type 16." *Journal of General Virology* 70.1 (1989): 69-77. Web.

Roden, Richard, and T-C. Wu. "How Will HPV Vaccines Affect Cervical Cancer?." *Nature Reviews Cancer* 6.10 (2006): 753-763.

Roseto, A., P. Pothier, M.-C. Guillemin, J. Peries, F. Breitburd, N. Bonneaud, and G. Orth. "Monoclonal Antibodies to the Major Capsid Protein of Human Papillomavirus Type 1." *Journal of General Virology* 65.8 (1984): 1319-324. Web.

Schiffman, Mark, et al. "Human Papillomavirus and Cervical Cancer." *The Lancet* 370.9590

(2007): 890-907.
Schwarz, Elisabeth, et al. "Structure and Transcription of Human Papillomavirus Sequences in Cervical Carcinoma Cells." (1985): 111-114.
Schellenbacher, Christina, Richard Roden, and Reinhard Kirnbauer. "Chimeric L1-L2 virus-like Particles as Potential Broad-spectrum Human Papillomavirus Vaccines." *Journal of Virology* 83.19 (2009): 10085-10095.
Smith, Jennifer S., et al. "Human Papillomavirus Type Distribution in Invasive Cervical Cancer and High-grade Cervical Lesions: A Meta-analysis Update." *International Journal of Cancer* 121.3 (2007): 621-632.
Smith, Kendall A., Margaret F. Favata, and Stephen Oroszlan. "Production and Characterization of Monoclonal Antibodies to Human Interleukin 2: Strategy and Tactics." *The Journal of Immunology* 131.4 (1983): 1808-1815.
Stewart, Bernard W., and Christopher P. Wild. *World Cancer Report 2014*. Geneva: World Health Organization, 2014. Print.
Tota, Joseph E., et al. "Epidemiology and Burden of HPV Infection and Related Diseases: Implications for Prevention Strategies." *Preventive Medicine* 53 (2011): S12-S21.
Walboomers, Jan MM, et al. "Human Papillomavirus is a Necessary Cause of Invasive Cervical Cancer Worldwide." *The Journal of Pathology* 189.1 (1999): 12-19.
Waldmann, Thomas A. "Monoclonal Antibodies in Diagnosis and Therapy." *Science* 252.5013 (1991): 1657-1662.
Yuan, Hang, et al. "Immunization with a Pentameric L1 Fusion Protein Protects Against Papillomavirus Infection." *Journal of Virology* 75.17 (2001): 7848-7853.